

Immune Profiling of Plasma and Cervical Secretions Using Recycling Immunoaffinity Chromatography

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Abstract

Small volumes of cervical secretions have limited measurements of immunity at the cervix, which may be important to studies of human papillomavirus (HPV). We report the use of recycling immunoaffinity chromatography to efficiently study immune profiles in cervical secretions. Frozen pairs of plasma and cervical secretions (collected on ophthalmic sponges) were selected randomly from women with normal cervical cytology ($n = 50$) participating in a natural history study of HPV in Guanacaste, Costa Rica. Single 25- μ l aliquots of plasma and (diluted) cervical secretions were assayed for interleukin (IL) -1 β , -2, -4, -6, -8, -10, -12, -13, -15, IFN- α , - β , - γ , tumor necrosis factor- α , - β , RANTES (regulated on activation normal T-cell express and secreted), MCP-1 (monocyte chemoattractant protein), -2, -3, macrophage inflammatory protein-1 α , -1 β (regulated on activation normal T-cell express and secreted), macrophage colony-stimulating factor, IgG, IgA, and cyclooxygenase 2. All of the specimens were tested as blind replicates, and refrozen plasma was retested 4 months later. To evaluate the reproducibility of the repeat measurements and to examine the correlation between plasma and cervical secretions, we calculated κ

values with 95% confidence intervals among categorized analyte values and Spearman correlation coefficients (ρ) among detectable, continuous analyte values.

Measurements of all of the analytes in either plasma or cervical secretions were highly reproducible, with all of the $\kappa \geq 0.78$ (70% above 0.90), and all of the $\rho \geq 0.88$ (96% above 0.90). Only IL-1 β ($\kappa = 0.60$ and $\rho = 0.82$) and IL-6 ($\kappa = 0.50$ and $\rho = 0.78$) levels were strongly correlated between plasma and cervical secretions. IFN- γ , tumor necrosis factor- β , RANTES, MCP-1, MCP-2, macrophage inflammatory protein-1 α , and macrophage colony-stimulating factor levels were especially poorly correlated between plasma and cervical secretions ($\kappa \leq 0.25$ and $\rho \leq 0.25$). We conclude that recycling immunoaffinity chromatography is a reproducible method of measuring immune profiles from biological specimens, and immune profiles are not well correlated between plasma and cervical secretions, perhaps necessitating cervical collections to study cervix-specific immunity in HPV natural history studies.

Introduction

Human papillomavirus (HPV), the most common sexually transmitted infection, is now recognized as the necessary cause of cervical cancer (1, 2). HPV infection is usually transient, but some infections persist and then can progress to cervical cancer and its immediate precursors (3). Host immune responses may be important in determining the outcome of infection (4–6). However, it is not yet clear what the molecular characteristics of an effective immune response for clearance of infection are. Such information may be valuable in developing therapeutic vaccines targeting HPV infection (7).

Epidemiological studies have typically relied on serological measurements of immunity to HPV, but HPV infections are localized to the lower genital tract and do not result in apparent viremia. Moreover, based on studies of a few immune biomarkers, blood concentrations of immune markers may be at best only modestly correlated with concentrations at the cervix (8–10). This is the likely consequence of independent, local production of these immune biomarkers at the cervix, which is part of the common mucosal immune system. Thus, serological measurements may not be fully representative of immune responses to HPV. However, the relationship between immunity at the cervix and systemic immunity has not been adequately described.

Measurements at the cervix have been limited by a combination of sampling methodology and by specimen volume. Cervicovaginal lavages represent an admixture of secretions of the cervix and vagina, and there is some evidence to suggest that there are immunological distinctions between these physiological sites (11, 12). Thus, measuring immunity at the cervix, where most HPV-induced cancers occur, may be more relevant than using a cervicovaginal lavage. Direct sampling of the

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cervical os circumvents these issues, but must avoid inducing bleeding that could contaminate secretions. Passive absorptive methods of collection, such as placing an ophthalmic-type sponge at the os of the cervix, appear optimal for examining local immunity and conveniently do not interfere with Pap screening (13, 14). However, the small volumes of these specimens (~50 μ l) typically permit only very limited numbers of analyte measurements leading to an incomplete description of cervical immunity.

Several proteomic methods have been developed recently, including protein microarrays (15), flow cytometric methods (16), and recycling immunoaffinity chromatography (RIC; Ref. 17), that might be used to provide a more extensive immune profile at the cervix and other mucosal surfaces. Protein microarrays, like ELISAs, are two-antibody systems with one antibody immobilized on the surface of the chip for analyte capture and a second antibody used for detection of the analyte. Flow cytometric methods (e.g., xMAP Technology; Luminex Corporation, Austin, TX) also use a two-antibody system with microparticles that combine a unique fluorescent emission with immunospecificity for each analyte. RIC is a single-antibody system that uses a set of high-performance liquid chromatographic immunoaffinity columns in series, with each column immunospecific for a targeted, fluorescently labeled analyte.

RIC has been shown to work on a number of biological specimens including a type of mucous secretion, saliva (17), and requires a specimen volume of 25 μ l. Thus, we were interested in examining whether RIC could reproducibly measure a panel of immune markers from plasma and cervical secretion collected on ophthalmic sponges, and determining how analyte concentrations found in cervical secretions would correlate with concentrations found in plasma. To address these questions, we selected 50 women with paired plasma and cervical secretions from a large cohort study of the natural history of HPV and cervical neoplasia in Guanacaste, Costa Rica (18–20). Both types of specimens were tested by RIC for a panel of representative markers of inflammation [interleukin (IL) -1 β , IL-6, IL-8, tumor necrosis factor (TNF) - α , TNF- β , IFN- α , IFN- β , cyclooxygenase (COX) 1, and COX-2], T-helper 1-like cytokines (IL-2, IFN- γ , IL-12, and IL-15), humoral immunity/T-helper 2-like cytokines (IL-4, IL-10, IL-13, IgG, and IgA), and chemokines [RANTES, MCP-1, MCP-2, MCP-3, macrophage inflammatory protein (MIP) -1 α , MIP-1 β , and macrophage colony-stimulating factor]. This panel was selected to cover broad patterns (immune profiles) related to disease development and immunological responses to HPV. Our aims in this exploratory study were to evaluate the reproducibility of this method before its application to critical specimens from our HPV natural history study, and to determine whether measurements of cervical secretions for evaluating HPV immunity were necessitated by poor correlations between cervix and blood for immune profiles.

Materials and Methods

Study Population and Specimen Selection. A National Cancer Institute-sponsored, National Cancer Institute- and local Institutional Review Board-approved population-based cohort study of HPV and cervical neoplasia was established in Guanacaste, Costa Rica, in 1993–1994 (18–20). At enrollment, 10,049 of the 11,742 women identified in a door-to-door survey residing in randomly chosen censal segments of Guanacaste agreed to visit one of our study clinics and participated in the enrollment interview. Pelvic examinations were performed on 9,175 women, excluding virgins ($n = 583$) and those women

unwilling or unable to undergo an exam ($n = 291$). At enrollment and during all of the follow-up visits, women undergoing pelvic exams were screened for cervical abnormalities using visual inspection, cytology, or cervicography, and women positive for any of these tests were referred to colposcopy for additional evaluation. Women participating in the cohort study were followed for 7 years.

Cervical secretions were collected using ophthalmic surgical sponges (Weck-cel; XOMED Surgical Products, Inc., Jacksonville, FL) at each follow-up visit after late 1996 for the following groups of women (20): (a) all women who had mild HPV-related cytologic abnormalities at enrollment that were untreated ($n = 155$); (b) a random subset of women who were HPV and cytologically negative, and who had <5 sexual partners lifetime ($n = 354$); (c) all originally virginal women after they reported their sexual debut ($n = 249$); (d) a single visit for a random sample of women who were under active follow-up ($n = 303$); and (e) all of the women with evidence of an incident case of high-grade cervical neoplasia not captured in the above sampling ($n = 93$). These groups were selected to pilot the collection of cervical secretions for immunological measurements, and included women both at low risk and high risk of developing cervical neoplasia, as well as all incident cervical neoplasia high-grade.

For this analysis, paired cervical secretions and plasma from 50 randomly selected untreated women who were negative for cytologic abnormalities (i.e., without atypical cells of unknown significance or low-grade squamous intraepithelial lesions) were tested. The median age of this group of women was 46 years (range, 23–71 years), and 71% were over the age of 40 years.

Data and Specimen Collection. At each visit, sexually active women who gave consent underwent a pelvic examination, at which time Pap smears were prepared, cells were collected for semiautomated ThinPrep cytology (Cytoc Corp., Boxborough, MA), and Cervigrams (National Testing Laboratories Worldwide, Fenton, MO) were taken. An additional cervical cell specimen was obtained with a dacron swab, which was placed in 1.0 ml of specimen transport medium (Digene Corporation, Gaithersburg, MD) and stored frozen until used for HPV DNA testing (18–20). A blood specimen was also collected by venipuncture into heparinized tubes, and the resultant plasma was aliquoted and stored first at -30°C and then at -70°C .

Cervical secretions were collected by placing a Weck-cel ophthalmic sponge gently in the cervical os, which was allowed to passively absorb cervical effluent for ~30 s as described previously (13, 14). Women were not examined and, therefore, secretions were not collected, during the menses. The study nurse (L. A. M.) was instructed to avoid touching the vaginal wall and vulva during collection with the sponge. Two specimens were collected sequentially from each woman before Pap smear sampling and stored first at 4°C , frozen at -30°C , and finally stored at -70°C until tested.

Extraction of Ophthalmic Sponges. Sponges were extracted using a protocol described previously (21). Briefly, each sponge was weighed to estimate the volume of secretions absorbed onto the sponge. The sponge was then treated in 300 μ l of extraction buffer (PBS, 0.25 M NaCl, 0.1 mg/ml aprotinin, and 0.001% sodium azide) and incubated for 30 min at 4°C . Sponges were then centrifuged at $16,000 \times g$ in a Spin-x centrifuge filter unit (Costar, Cambridge, MA) to separate extracted samples from the sponge matrix. The sponge was again treated with an additional 300 μ l of extraction buffer and immediately centrifuged in the Spin-X tube. The two extraction

volumes were combined, 10 μ l was removed for hemoglobin testing by Hemastix (VWR International, West Chester, PA), and then 4 μ l FCS was added to the remaining specimen. A dilution factor was calculated based on the estimated volume and dry weight of the spear as described previously (6): $[(x - y) + 0.6 \text{ g of buffer}]/(x - y)$, where x equals the weight of the sponge after collection, and y is the weight of the dry spear.

RIC Testing. RIC was performed as described previously (17, 22). Briefly, all of the cytokines and their corresponding reactive antibodies were obtained from R & D Systems (Minneapolis, MN). Human IgG, IgA, and serum albumin, and their corresponding reactive antibodies were purchased from Sigma-Aldrich (St. Louis, MO), and COX-1 and -2 proteins plus their reactive antibodies were purchased from α Diagnostic International, Inc. (San Antonio, TX). Stock solutions of both antibodies and their respective antigens were adjusted to 1 μ g/ml in 100 mM phosphate (pH 7.4). Fc portions of each antibody biotinylated using the hydrazine-biotin reaction (23). Specimens were labeled with AlexaFluor 633 fluorochrome (Molecular Probes, Eugene, OR) as described previously (24).

Acid-washed glass beads ($\sim 10 \mu$ m diameter; Polysciences, Warrington, PA) were derivatized with 3-aminopropyltriethoxy-silane (ICN Biomedicals, Costa Mesa, CA) and then 1,1'-carbodiimidazole (ICN), and streptavidin (Pierce Chemical Co.) were covalently linked to the beads. Biotinylated antibodies were immobilized to the streptavidin beads by incubating the antibodies and beads together overnight at 4°C. After washing, the beads were loaded into 4.6 mm ID \times 25-mm long PEEK columns (Alltech Associates, Deerfield, IL), and the columns were attached to an automated high-performance liquid chromatography system (Alltech Associates) equipped with a programmable 10-column selector (17, 24).

An aliquot of each fluorochrome-labeled sample (25 μ l) was injected into the system, which was programmed to pass the sample through each column in series. Ten columns were connected to the selector, enabling recycling of each sample serially through all of the columns; the effluent from the last column was collected and loaded onto the next panel of columns with immunospecificities for a different set of analytes. Introducing 0.33 M citric acid into the running buffer, to produce a 7.4–1.0 pH gradient and programming the system to acid-elute each column individually in parallel, achieved recovery of each bound analyte. The eluted analytes were detected using an on-line, laboratory-built laser-induced fluorescence detector. The signal from this detector was relayed to a CCD-based spectrometer (SF2000; Ocean Optics, Inc., Dunedin, FL) interfaced with computer, which used the spectrometer manufacturers software (OOIBase 32; Ocean Optics). Dilutions of recombinant immune markers solutions (standards) were used to develop standard curves for estimation of analyte concentrations. Throughout the study, the sequence of immunoaffinity columns were maintained to control for any differences due to the ordering of the columns.

Statistical Analysis. Of the 50 paired specimens selected, there was 1 missing plasma specimen. Therefore, 50 extracted cervical secretions and 49 plasma specimens were tested. Two aliquots of each specimen were placed into separate tubes, and the aliquots were tested as masked duplicates to examine intrabatch reproducibility. Approximately 4 months later, using newly prepared batches of immunoaffinity beads, the residual refrozen plasmas were tested again, masked to the original test result to examine interbatch reproducibility. One of the 49 plasma specimens was not tested in duplicate during the original testing, and so there were only 48 repeat tests to evaluate

intrabatch reproducibility; all 49 of the plasma specimens were retested for interbatch reproducibility. Two cervical secretion specimens were not tested in duplicate, and so there were only 48 tests to evaluate intrabatch reproducibility.

Specimens were tested for the following analytes: IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IFN- α , IFN- β , IFN- γ , TNF- α , TNF- β , RANTES, MCP-1, MCP-2, MCP-3, MIP-1 α , MIP-1 β , macrophage colony-stimulating factor, IgG, IgA, human serum albumin, COX-1, and COX-2. Limits of detection were 2 pg/ml for all of the markers except COX-1 and COX-2, which had a detection limit of 250 ng/ml, IgA and IgG, which had a limit of detection of 2 μ g/ml, and human serum albumin, which had a limit of detection of 2 mg/ml. COX-1 was detectable in only 5 cervical specimens and 3 plasma specimens, and, therefore, could not be evaluated in this study. The mean concentration, median concentration, and range of concentrations were determined for each biomarker from each specimen.

To evaluate the reproducibility of the repeat measurements and to examine the correlation between plasma and cervical secretions, κ values with 95% confidence intervals and Spearman correlation coefficients (ρ) were calculated. For κ analyses, categories of below the limits of detection, lowest tertile, mid-tertile, or upper tertile for each analyte (except COX-2) and for each type of specimen (cervical secretion or plasma) were defined. Categories were based on the distribution of values of the first set of tests. COX-2 was categorized as below the limits of detection or detectable. κ values were arbitrarily assigned a classification similar to the one used by Byrt (25): poor (0.0 to <0.2), slight (0.2 to <0.4), fair (0.4 to <0.6), good (0.6 to <0.8), and excellent agreement (0.8–1.0). Spearman correlations were calculated among detectable levels only. For correlations of cervical secretion with plasma, 1 pair did not

Table 1 Summary of measurements of plasma analytes

	Plasma levels (n = 49)				
	% ^a	Mean	Median	Range	Units
IL-1 β ^b	100%	31.0	16.3	4.5–113.4	pg/ml
IL-2	100%	13.4	8.8	2.4–75.2	pg/ml
IL-4	100%	12.7	10.4	2.2–51.9	pg/ml
IL-6	100%	29.3	14.1	4.3–101.3	pg/ml
IL-8	63%	11.7	9.7	2.2–36.2	pg/ml
IL-10	100%	9.1	6.5	2.1–41.6	pg/ml
IL-12	100%	9.7	6.9	2.4–44.7	pg/ml
IL-13	88%	9.9	7.7	2.1–36.4	pg/ml
IL-15	22%	19.4	14.9	6.7–44.8	pg/ml
IFN- α	94%	10.7	6.4	2.1–61.7	pg/ml
IFN- β	98%	8.0	6.0	2.2–49.9	pg/ml
IFN- γ	100%	8.2	6.2	2.1–30.6	pg/ml
TNF- α	100%	15.0	7.3	2.5–98.6	pg/ml
TNF- β	35%	10.7	10.4	2.9–23.2	pg/ml
RANTES	39%	13.1	10.4	2.1–37.3	pg/ml
MCP-1	37%	12.7	8.6	3.3–40.9	pg/ml
MCP-2	39%	12.1	9.5	2.4–29.6	pg/ml
MCP-3	39%	12.8	10.2	2.3–38.2	pg/ml
MIP-1 α	37%	14.8	10.1	2.2–45.6	pg/ml
MIP-1 β	37%	14.4	8.5	2.1–58.2	pg/ml
M-CSF	98%	14.1	10.2	2.5–80.8	pg/ml
IgG	100%	12.2	11.1	8.4–19.2	mg/ml
IgA	100%	2.0	1.7	1.3–3.7	mg/ml
HSA	100%	37.0	37.9	14.6–60.4	mg/ml
COX-2	6%	161.3	142.8	23.9–317.2	ng/ml

^a Percentage of specimens with detectable levels.

^b IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; M-CSF, macrophage-colony-stimulating factor; COX, cyclooxygenase.

Table 2 Reproducibility of plasma analytes by recycling immunoaffinity chromatography evaluated by Spearman correlation coefficients (ρ) and κ values with 95% confidence intervals (CIs)

	Intra-assay ($n = 48$)				Interassay ($n = 49$)			
	% ^a	ρ^b	κ^c	95% CI	% ^a	ρ^b	κ^c	95% CI
IL-1 $\beta^{d,e}$	100%	1.00	0.91	0.72–1.00	100%	1.00	0.94	0.74–1.00
IL-2	100%	0.99	0.85	0.66–1.00	100%	1.00	0.85	0.65–1.00
IL-4	100%	1.00	0.91	0.71–1.00	100%	1.00	0.88	0.69–1.00
IL-6	100%	1.00	0.85	0.65–1.00	100%	1.00	0.85	0.65–1.00
IL-8	63%	0.99	0.94	0.78–1.00	65%	0.99	0.94	0.78–1.00
IL-10	100%	0.99	0.91	0.71–1.00	96%	0.99	0.85	0.67–1.00
IL-12	100%	0.99	0.97	0.78–1.00	100%	0.99	0.94	0.75–1.00
IL-13	88%	0.99	0.85	0.65–1.00	88%	0.99	0.80	0.64–1.00
IL-15	23%	0.99	1.00	0.79–1.00	23%	1.00	0.89	0.69–1.00
IFN- α	94%	0.99	0.91	0.74–1.00	96%	0.97	0.91	0.73–1.00
IFN- β	98%	0.99	0.88	0.69–1.00	98%	0.99	0.85	0.66–1.00
IFN- γ	100%	0.99	0.82	0.63–1.00	100%	0.99	0.88	0.68–1.00
TNF- α	100%	1.00	0.82	0.63–1.00	100%	0.99	0.82	0.62–1.00
TNF- β	35%	1.00	0.92	0.74–1.00	35%	0.99	0.96	0.78–1.00
RANTES	40%	1.00	0.89	0.72–1.00	40%	0.99	0.89	0.72–1.00
MCP-1	38%	0.99	0.89	0.71–1.00	38%	0.98	0.96	0.78–1.00
MCP-2	40%	0.98	0.96	0.79–1.00	40%	0.98	0.96	0.79–1.00
MCP-3	40%	0.99	1.00	0.82–1.00	40%	0.99	0.96	0.79–1.00
MIP-1 α	38%	0.99	0.93	0.75–1.00	38%	0.94	0.89	0.71–1.00
MIP-1 β	35%	0.88	0.85	0.67–1.00	38%	0.90	0.85	0.67–1.00
M-CSF	98%	0.99	0.91	0.72–1.00	100%	1.00	0.94	0.75–1.00
IgG	100%	1.00	0.97	0.78–1.00	100%	1.00	0.97	0.77–1.00
IgA	100%	1.00	0.97	0.78–1.00	100%	1.00	0.97	0.77–1.00
HSA	100%	1.00	0.94	0.74–1.00	100%	1.00	0.97	0.77–1.00
COX-2 ^e	6%	1.00	1.00	0.72–1.00	6%	1.00	1.00	0.72–1.00

^a Percentage of repeated tests with detectable levels for both tests.^b Spearman correlation coefficients were calculated among samples with detectable levels.^c Categories of below limits of detection (BLD), low tertile, medium tertile, and high tertile were used.^d IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; M-CSF, macrophage-colony-stimulating factor; COX, cyclooxygenase.^e For κ analysis, categories of BLD and detectable levels were used.

have a plasma specimen, and 4 cervical secretions did not have estimated volumes because of missing dry weights for that production lot of ophthalmic sponges. Thus, only 45 pairs were used for examining correlations between plasma and cervical secretions (values of all 50 cervical secretions were used because adjustment for dilution volume is not needed for calculating the reproducibility of cervical secretion measurements).

To examine the effects of covariates on the correlations between plasma and cervical analytes, we stratified the plasma/cervical secretion correlations (Spearman) on hemoglobin status (negative or positive; only 1 specimen had more than trace amounts of hemoglobin), median volume of cervical secretions ($<54 \mu\text{L}$ or $\geq 54 \mu\text{L}$), and median age at the time of collection (<48 years or ≥ 48 years; 48 years was the median age for the subset of 45 women with complete data) for those analytes for which $>80\%$ pairs had detectable levels for both specimens: IL-1 β , IL-6, IL-10, IL-12, IFN- β , TNF- α , IgG, and IgA. These covariates have been reported previously as determinants for cervical secretion concentrations (13). Although previous studies have also demonstrated the importance of time of menstrual cycle and hormonal contraceptive use on the concentrations of cervical analytes, we were unable to examine these factors because of the small numbers ($n = 22$) of women who were menstruating at the time of collection or were using hormonal contraception ($n = 8$). Differences in Spearman correlation coefficients between strata were tested for statistical significance ($P < 0.05$) by calculating the Z statistic using Fisher's z transformation of correlation coefficients (26).

Results

The results of plasma testing by RIC are summarized in Table 1. IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IFN- α , IFN- β , IFN- γ , TNF- α , macrophage colony-stimulating factor, IgG, IgA, and human serum albumin were detected in $>90\%$ of the specimens tested. The median concentrations of IgG and IgA were 11.8 mg/mL and 1.9 mg/mL, respectively. COX-2 was only detectable in 3 of 49 plasma specimens but was more often detectable in cervical secretions (see below).

RIC testing of plasma was highly reproducible within a test batch (intrabatch) and between test batches (interbatch; Table 2). Among specimens in which both tests for an analyte were above the limit of detection, the intrabatch Spearman coefficients for all of the analytes were ≥ 0.98 except for MIP-1 α , which had a coefficient of 0.88. Intrabatch κ values, which factored those values below detection limits into the calculations of reproducibility, were excellent (≥ 0.80) for all of the analytes. To additionally verify the reliability of the RIC assay, we retested the same (refrozen) aliquot 4 months later. All of the interbatch Spearman coefficients were ≥ 0.98 except for MIP-1 α , which had a coefficient of 0.94. Interbatch κ values were also excellent (≥ 0.80) for all of the analytes. The small differences in Spearman correlation coefficients and κ values reflected those few specimens with immune marker concentrations near the limits of detection, with one measure above the limit and the other measure below the limit.

The results of testing cervical secretions are summarized in Table 3; values were adjusted for the dilution that resulted from extracting cervical secretions from the ophthalmic

Table 3 Summary of measurements of cervical secretion analytes

Cervical levels ^a (n = 45 specimens)					
	% ^b	Mean	Median	Range	Units
IL-1 β ^c	100%	4,270.7	1,341.4	(73.3–32,011.0)	pg/ml
IL-2	80%	667.0	412.1	(48.6–3,583.5)	pg/ml
IL-4	51%	253.5	176.9	(28.7–877.9)	pg/ml
IL-6	100%	3,765.1	1,384.8	(59.3–28,722.6)	pg/ml
IL-8	91%	1,191.0	454.5	(31.6–6,393.1)	pg/ml
IL-10	93%	272.3	175.3	(24.1–1,965.7)	pg/ml
IL-12	89%	696.7	357.1	(24.9–4,004.1)	pg/ml
IL-13	84%	733.8	293.4	(33.1–3,000.0)	pg/ml
IL-15	60%	1,040.1	630.2	(10.3–4,997.9)	pg/ml
IFN- α	60%	1,942.1	1,161.4	(15.6–8,109.5)	pg/ml
IFN- β	87%	2,078.6	458.0	(61.6–17,459.3)	pg/ml
IFN- γ	47%	561.6	270.8	(11.7–4,090.1)	pg/ml
TNF- α	100%	5,060.2	1,696.4	(124.5–43,313.4)	pg/ml
TNF- β	51%	443.0	255.8	(53.9–32,39.6)	pg/ml
RANTES	71%	907.7	451.7	(36.2–4,290.8)	pg/ml
MCP-1	73%	638.6	402.0	(44.2–3,526.3)	pg/ml
MCP-2	64%	588.8	365.9	(51.7–3,019.8)	pg/ml
MCP-3	67%	709.4	485.3	(42.7–2,591.0)	pg/ml
MIP-1 α	67%	641.6	366.2	(39.2–3,567.9)	pg/ml
MIP-1 β	67%	546.0	432.2	(33.6–1,767.9)	pg/ml
M-CSF	76%	1,206.0	703.5	(12.7–7,606.8)	pg/ml
IgG	100%	0.9	0.4	(0.01–4.3)	mg/ml
IgA	100%	1.5	0.5	(0.05–8.1)	mg/ml
HSA	100%	545.5	143.5	(9.5–6,546.0)	mg/ml
COX-2	33%	2,725.8	1,311.3	(58.8–10,750.8)	ng/ml

^a Values adjusted for the dilution as the result of extracting secretions from the ophthalmic sponge.

^b Number of specimens with detectable levels.

^c IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; M-CSF, macrophage-colony-stimulating factor; COX, cyclooxygenase.

sponges. IL-1 β , IL-6, IL-8, IL-10, TNF- α , IgG, IgA, and human serum albumin were detectable in >90% of the specimens tested. Median concentrations of IgG and IgA were 370.3 μ g/ml and 573.4 μ g/ml, respectively. COX-2 was detectable in 15 of 45 specimens (33%) in which we are able to estimate the dilution due to extraction from the sponges [overall, 18 of 50 specimens tested (36%) had measurable COX-2 levels].

RIC testing of masked duplicate aliquots of cervical secretions demonstrated that testing of cervical secretions was as reproducible as the testing of plasma (Table 4). Among positive tests for both aliquots, only MIP-1 α (ρ = 0.90) had an intrabatch Spearman coefficient <0.98. All of the intrabatch κ values were \geq 0.85 except for IgA, which had a κ value of 0.79.

The correlations of these analytes comparing plasma with cervical secretions are shown in Table 5. Among paired plasma and cervical secretions with detectable levels of analytes, IL-1 β had the highest Spearman correlation coefficient of 0.82, and IL-2, IL-6, and IFN- α had coefficients of \geq 0.60. IFN- γ , TNF- α , and MCP-2 were not correlated. Only IL-1 β (κ = 0.60) and IL-6 (κ = 0.50) had κ values >0.40.

We examined the effects of a few covariates on the relationship between plasma and cervical concentrations of several analytes (IL-1 β , IL-6, IL-10, IL-12, IFN- β , TNF- α , IgG, and IgA) selected because the levels were detectable in >80% of the specimens (Table 6). Plasma and cervical IL-12 was significantly less correlated (P < 0.05) in paired specimens in which hemoglobin was present in cervical secretions (ρ = -0.01) compared with those in which it was absent (ρ = 0.73), in cervical specimens with less than the median volume (ρ = 0.05) compared with those with the median volume or greater (ρ = 0.81), and from women aged 48 years and older (ρ = 0.21)

compared with women under the age of 48 (ρ = 0.74; P < 0.05) for all comparisons. The effects of three covariates on IL-12 appeared independent because they were not found to be associated with each other (data not shown). Cervical secretions with hemoglobin present had poorer correlations for all of the selected analytes, but these differences did not reach statistical significance except for IL-12.

Discussion

We have demonstrated that RIC can reproducibly determine multiple cytokines, inflammation-related enzymes, and immunoglobulin levels in biological fluids including cervical secretions, which typically have limited volumes (in this study, the estimated median volume was 54 μ l, and the range was 6–154 μ l). Overall, immune profiles of plasma and cervical secretions were only slightly correlated among a group of women without overt infection or disease. For infections localized to cervix, such as HPV infection, we anticipate a greater distinction between the concentrations of immune markers in plasma and in cervical secretions, which will more directly reflect the local immune response to infection.

It is still uncertain how accurately an immune profile in cervical secretions reflects immunity in the cervical tissue. A recent study (27) found no correlations between relative mRNA levels and cytokine protein concentrations in cervicovaginal lavage cell pellet and supernatant, respectively. However, that

Table 4 Reproducibility of cervical secretion analytes by recycling immunoaffinity chromatography evaluated by Spearman correlation coefficients (ρ) and κ values with 95% confidence intervals (CIs)

Intra-assay (n = 48)				
	% ^a	ρ ^b	κ ^c	95% CI
IL-1 β	100%	1.00	0.94	0.75–1.00
IL-2	83%	1.00	0.97	0.81–1.00
IL-4	54%	0.99	0.85	0.68–1.00
IL-6	100%	1.00	0.85	0.66–1.00
IL-8	92%	1.00	0.92	0.75–1.00
IL-10	94%	1.00	0.92	0.74–1.00
IL-12	92%	1.00	0.97	0.80–1.00
IL-13	85%	1.00	0.95	0.78–1.00
IL-15	65%	1.00	0.97	0.81–1.00
IFN- α	63%	1.00	0.97	0.81–1.00
IFN- β	92%	1.00	0.86	0.70–1.00
IFN- γ	50%	1.00	0.97	0.80–1.00
TNF- α	100%	1.00	0.91	0.72–1.00
TNF- β	54%	1.00	1.00	0.83–1.00
RANTES	75%	0.98	0.92	0.76–1.00
MCP-1	73%	1.00	0.95	0.79–1.00
MCP-2	67%	1.00	0.95	0.78–1.00
MCP-3	67%	1.00	0.95	0.78–1.00
MIP-1 α	69%	1.00	0.95	0.78–1.00
MIP-1 β	69%	0.90	1.00	0.84–1.00
M-CSF	73%	1.00	0.89	0.73–1.00
IgG	100%	0.98	0.88	0.69–1.00
IgA	100%	0.98	0.79	0.61–1.00
HSA	100%	1.00	0.94	0.75–1.00
COX-2 ^e	38%	1.00	1.00	0.82–1.00

^a Number of pairs with detectable levels.

^b Spearman correlation coefficients were calculated among samples with detectable levels.

^c Categories of below limits of detection (BLD), low tertile, medium tertile, and high tertile were used.

^d IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; M-CSF, macrophage-colony-stimulating factor; COX, cyclooxygenase.

^e For κ analysis, categories of BLD and detectable levels were used.

Table 5 The relationships of cervical and plasma concentrations of analytes evaluated by Spearman correlation coefficients and κ values with 95% confidence intervals (95% CI)

	Correlations of cervical ^a and plasma levels					
	<i>n</i> (cervical) ^b	<i>n</i> (plasma) ^b	<i>n</i> (pairs) ^b	ρ^c	κ^d	95% CI
IL-1 β^e	45	45	45	0.82	0.60	0.39–0.81
IL-2	36	45	36	0.61	0.09	–0.08–0.26
IL-4	23	45	23	0.21	0.09	–0.03–0.21
IL-6	45	45	45	0.78	0.50	0.29–0.71
IL-8	41	27	27	0.39	0.15	0.00–0.30
IL-10	42	45	42	0.53	0.10	–0.09–0.29
IL-12	40	45	40	0.47	0.18	0.00–0.36
IL-13	38	39	33	0.43	0.12	–0.05–0.30
IL-15	27	9	9	0.40	0.10	–0.04–0.24
IFN- α	27	42	27	0.62	0.24	0.10–0.38
IFN- β	39	44	39	0.50	0.16	–0.02–0.34
IFN- γ	21	45	21	–0.04	0.05	–0.06–0.16
TNF- α	45	45	45	0.51	0.30	0.09–0.51
TNF- β	23	14	12	–0.21	0.24	0.06–0.41
RANTES	32	17	17	0.11	0.24	0.09–0.39
MCP-1	33	16	16	0.20	0.13	–0.02–0.28
MCP-2	29	17	17	–0.07	0.17	0.01–0.33
MCP-3	30	17	17	0.32	0.28	0.12–0.44
MIP-1 α	30	16	16	0.11	0.21	0.06–0.37
MIP-1 β	30	16	16	0.43	0.31	0.15–0.46
M-CSF	34	44	34	0.24	0.14	–0.02–0.30
IgG	45	45	45	0.55	0.23	0.03–0.44
IgA	45	45	45	0.41	0.17	–0.04–0.37
HSA	45	45	45	–0.58	–0.20	–0.40–0.01
COX-2 ^f	15	2	2	–1.00	0.17	0.01–0.33

^a Values adjusted for the dilution as the result of extracting secretions from the ophthalmic sponge.

^b Number of specimens with detectable levels.

^c Spearman correlation coefficients were calculated among samples with detectable levels.

^d Categories of below limits of detection (BLD), low tertile, medium tertile, and high tertile were used.

^e IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; M-CSF, macrophage-colony-stimulating factor; COX, cyclooxygenase.

^f For κ analysis, categories of BLD and detectable levels were used.

study was small ($n = 24$) and differed methodologically from this study by using a cervicovaginal lavage. In this study, cervical levels of inflammatory biomarkers IL-1 β , IL-6, IL-8, IFN- γ , and COX-2 were also associated with numbers of neutrophils on Pap smear slides ($P_{\text{Trend}} < 0.05$), but neither plasma concentrations of these markers nor cervical T-helper 2 immune markers (e.g., IL-4 and IL-10) were, suggesting that these molecular measurements of cervical inflammation were better correlated with gross inflammatory processes observable at the cervix (data not shown). Although we hypothesize that measurements in cervical secretions will more closely approximate the immune responses in cervical tissue than will plasma measurements, more studies are needed to examine these relationships.

Absolute values of these analytes in plasma vary widely between studies, perhaps as the result of different study populations and different assay methodology. We note that the plasma concentrations of IgG and IgA measured by RIC were consistent with previously accepted values of 9.5–12.5 mg/ml for IgG and 1.5–2.6 mg for IgA (28). IL-10 concentrations (mean = 9.1 pg/ml) in this study are similar to those reported previously in whole blood from normal women as tested by RIC (mean = 10 pg/ml; Ref. 17), and those reported in sera from fertile (mean = 13.5 pg/ml) and menopausal (mean = 16.0 pg/ml) women as tested by ELISA (29). IL-12 concentra-

tions (mean = 9.1 pg/ml) in this study were similar to those in whole blood from normal women as tested by RIC (mean = 14 pg/ml), and were somewhat greater than those recorded for fertile (mean = 2.4 pg/ml) and menopausal (mean = 2.6 pg/ml) women as tested by ELISA (23).

There are very few published studies with analyte concentrations in cervical secretions. A previous study using the same ophthalmic sponges and in the same population reported median values of 1 mg/ml IgG, 0.33 mg/ml IgA, 84.8 pg/ml IL-10, and 254.7 pg/ml IL-12 as measured by ELISA (13). A study examining the fluctuations during the menstrual cycle found a range of mean concentrations from 0.20 to 0.26 mg/ml IgG, 0.14 to 0.41 mg/ml IgA, ~400 to ~1000 pg/ml IL-1 β , ~300 to ~500 pg/ml IL-6, and ~10 to ~40 pg/ml IL-10 pg/ml for cervical secretions collected by aspiration (30). A study of adolescent women found mean concentrations of 34.5 pg/ml IL-10, 85.1 pg/ml IL-12, and 390.4 pg/ml IL-2 (31) in cervical secretions collected by ophthalmic sponges. Greater IL-10 and IL-12 concentrations in this study may also reflect differences in the assay methodologies used.

The correlations of IL-10 ($\rho = 0.53$) and IL-12 ($\rho = 0.47$) between plasma and cervical secretions in this study were better than those we observed previously for IL-10 ($\rho = 0.11$) and IL-12 ($\rho = -0.04$; Ref. 10). Specimens selected for this study were from women without overt cervical abnormalities compared with women in the previous study, who were enrolled into the study because of an equivocally abnormal Pap smear suggestive of an HPV infection.

Hemoglobin is an indicator of local bleeding during the collection although not necessarily because of the collection. Although we have selected a method of collection that is minimally invasive, it is unlikely that any method can be completely noninvasive, especially in this population with a high prevalence of cervicitis. Thus, the presence of blood remains an important modifier of genital tract immunological measurements, as previously observed (13), and must be accounted for in these analyses.

In this study, cervical specimens with hemoglobin had both lower median concentrations for all analytes (data not shown), even for IgG, which had a greater concentration in plasma, and poorer correlations between plasma and cervical secretions for all of the selected analytes compared with cervical secretions without hemoglobin. Hemoglobin status and volume of cervical secretions, and the age of the woman independently (i.e., variables were statistically unrelated to one another) modified the relationship between plasma and cervical secretions for IL-12. However, we emphasize that because of the small numbers in these strata, some of these differences may be due to chance alone, and that larger studies are needed to more thoroughly examine these relationships.

The mechanisms by which plasma contributes to cervical concentrations of analytes are not well understood. Given the significantly lower concentrations of all of the analytes except immunoglobulins in plasma *versus* cervical secretions, the fractional contribution from plasma cannot be explained by simple passive influx from plasma to the cervix but is suggestive of an active, physiological transport that is concentration dependent. Higher concentrations of immunoglobulins in plasma than in cervical secretions may suggest an alternative mechanism of passive transport down the concentration gradient. It is of note that the ratio of anti-HPV16 IgG concentration in cervical secretion *versus* in serum in women vaccinated with HPV16 L1

Table 6 Plasma/cervical Spearman correlation coefficients of select cytokines stratified on detection of hemoglobin and volume of cervical secretions, and the age of the woman at the time of specimen collectionStatistical significant differences between strata ($P < 0.05$) are indicated in bold, and n represents the maximum number of values within a stratum.

	Overall	Hemoglobin		Volume		Age (years)	
		No ($n = 26$)	Yes ($n = 19$)	$\leq 54 \mu\text{l}$ ($n = 22$)	$\geq 54 \mu\text{l}$ ($n = 23$)	< 48 ($n = 22$)	≥ 48 ($n = 22$)
IL-1 β^a	0.82	0.82	0.68	0.78	0.85	0.80	0.77
IL-6	0.78	0.82	0.69	0.80	0.74	0.66	0.86
IL-10	0.53	0.53	0.32	0.60	0.57	0.46	0.48
IL-12	0.47	0.73	-0.01	0.05	0.81	0.74	0.21
IFN- β	0.50	0.63	0.24	0.50	0.46	0.62	0.27
TNF- α	0.51	0.65	0.18	0.42	0.54	0.66	0.35
IgG	0.55	0.57	0.31	0.54	0.56	0.56	0.53
IgA	0.41	0.30	0.38	0.32	0.54	0.40	0.32

^a IL, interleukin; TNF, tumor necrosis factor.

virus-like particle vaccines⁶ was similar (~7%) to the ratio of nonspecific IgG in the cervical secretions *versus* plasma in this analysis, additionally suggesting some validity to our measurements using RIC.

We acknowledge two limitations of this study. First, the study was too small to statistically adjust for the important determinants of cervical concentrations of immune markers such as collection volume, age, time of the menstrual cycle, and oral contraceptive use (13), all of which are interrelated. In particular, it will be important to take oral contraceptive use into account in future work, given the possibly role of oral contraceptives in the development of cervical cancer (32). Second, there is *in vitro* evidence to suggest that some immune markers, particularly IL-4 and IFN- γ , are poorly recovered from Weck-cel ophthalmic sponges (21), perhaps due to irreversible binding to the sponge matrix. It is unclear whether this might also occur with cervical secretions, given the complex nature of the biospecimen with other proteins in high concentrations (e.g., albumin) that might block this undesirable binding. If this phenomenon does impact recovery of certain immune markers, we would expect that some measurements would be below the limits of detection and, therefore, artificially decrease the correlations between the plasma and cervical secretions. We are currently evaluating alternative ophthalmic sponges to overcome this limitation.

We conclude that RIC may be a valuable tool for the measurement of markers of cervical immunity related to HPV infection, but additional studies are needed. The small specimen volume requirement (25 μl) may permit the use of smaller extraction volumes for the ophthalmic sponges, reducing the dilution factor, and thereby more accurately describing the immune profile by increasing the percentage of detection for a given analyte. The versatility of this method will permit quantitative measurements of other analytes, such as COX-2, a glucocorticoid-regulated COX isoform that is induced in response to inflammation for the production of prostaglandins. COX-2 expression has been linked to cervical cancer (33). We note that COX-2 was detectable in 1 plasma specimen among the 15 cervical secretion specimens in which COX-2 was detectable, highlighting the potential importance of local measurements of biomarkers. Indeed, this method could be expanded to measure pathogen-specific antibodies, provided the antigen can be covalently attached to the glass beads. Miniaturization of the RIC method (34) will potentially increase the number of

analytes detected and will provide greater throughput that is required for epidemiological studies. Other methods such as flow cytometric methods, if validated, may also provide greater throughput. Finally, we emphasize that immunity in blood may at best be only modestly related to immunity at the cervix and that the continued development of proteomic methods such as RIC may provide a more comprehensive description of immune responses, both of which may be particularly relevant in the effort to understand HPV natural history.

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